

Expression Screening and Thermal Stability of Human Connexin 26

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Introduction

Two barriers to membrane protein structure analysis are protein expression and optimization of the protein detergent complexes. To help overcome these barriers in an efficient way, we used microscale expression to test a large number of mutants of the gap junction protein connexin26 (Cx26) and have begun examining the thermal stability of purified protein in detergent micelles using a fluorescence assay.

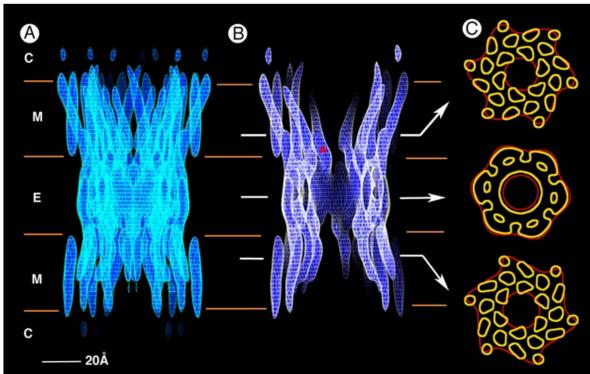
Background: Gap junction channels allow direct cell-to-cell movement of ions and signaling molecules to control the metabolic and electrical activities within tissues. The channel is formed by the end-to-end docking of two hemi-channels, each comprised of 6 connexin subunits. Each subunit contains 4 transmembrane domains (M1 to M4), and electron crystallography revealed that the hemichannel is formed by an annular bundle of 24 alpha-helices. Tight packing of the extracellular loops bridges the gap between cells and prevents exchange of molecules with the extracellular environment. Connexin 26 (Cx26) is one of the smallest family members, mutations of which are a common cause of nonsyndromic deafness.

Micro-scale expression: Wild-type, His-tagged Cx26 is expressed at high levels in insect cells using a recombinant baculovirus vector. An extensive series of truncations of the termini and loops were screened by micro-scale expression using the Thompson Vertiga IM incubator/shaker. Expression was maintained for truncations involving the M2-M3 intracellular loop (IL) and the second extracellular loop (EL2) between M3 and M4. However, mutants involving truncations of the N- and C-termini and the first extracellular loop (EL1) displayed markedly reduced levels of expression. This study has provided a number of useful constructs as well as guiding future avenues to optimize expression.

Thermal stability measurements: A simple fluorescence assay that uses microgram quantities of protein was used to measure the thermal stability of Cx26. Upon thermal denaturation a cysteine in the fourth transmembrane domain (C202) becomes accessible for labeling by a thio-reactive fluorescent dye which upon binding undergoes a large increase in quantum yield. Cx26 displays a denaturation profile in dodecylmaltoide suggesting a simple 2-state cooperative transition. Additional conditions are currently being tested.

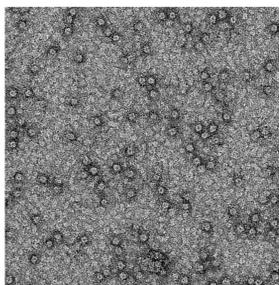
We are encouraged that the expression levels for several Cx26 mutants are sufficient for structural studies. The microscale expression and stability assay will be valuable for optimization of mutant, detergent, buffer and additive conditions to accelerate crystallization trials.

3D Structure of a Recombinant Gap Junction Channel by Electron Cryocrystallography



Unger et al., Science 283, 1176-1180 (1999).

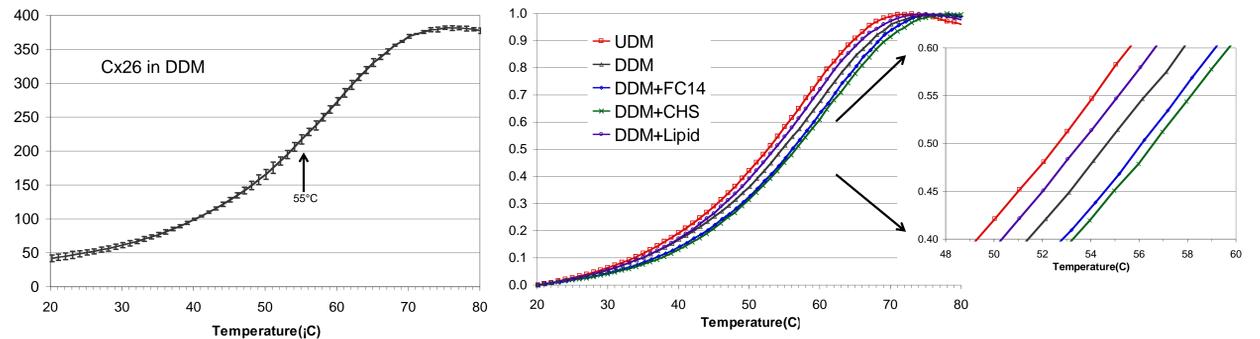
EM of recombinant Cx26



Negative-stain EM shows that recombinant, His-tagged Cx26 expressed in insect cells assembles as a hexameric. Courtesy of Kelly Dryden.

Intercellular gap junction channels have a diameter of ~65 Å and are formed by the end-to-end docking of two hexameric hemichannels called connexons. Each Cx43 subunit contains 4 transmembrane helices, so that the hemichannel is formed by a bundle of 24 helices. (a) side view and (b) cropped density to show the interior. The approximate boundaries for the membrane bilayers (M), extracellular gap (E) and the cytoplasmic space (C) are indicated. The extracellular wall of the channel is bounded by a continuous wall of protein, which forms a tight seal to prevent exchange of substances with the extracellular milieu. White arrows identify the locations of the cross sections (c) that are parallel to the membrane bilayers. The red contours define the outer boundary, and the yellow contours show the locations of the 24 helices within each membrane. Red asterisk identifies the narrowest part of the channel, which has a diameter of ~15Å.

Thermal Stability: Cx26 is least stable in UDM and most stable in DDM+CHS



Cooperative unfolding revealed by fluorescence:

In an effort to optimize the protein detergent complex for 3D crystallization, we measured the thermal stability of hexameric Cx26 in several detergents. Thermal stability is measured using a simple dye accessibility assay in which a small amount of thiol-reactive dye is added to the protein sample and then heated (Alexandrov et al. *). A single reactive cysteine in TM helix 4 (C202) becomes accessible upon unfolding, with an associated increase in the fluorescence intensity.

Methods:

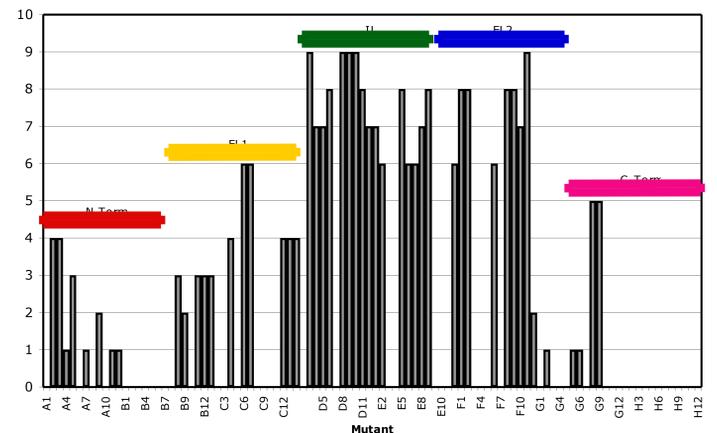
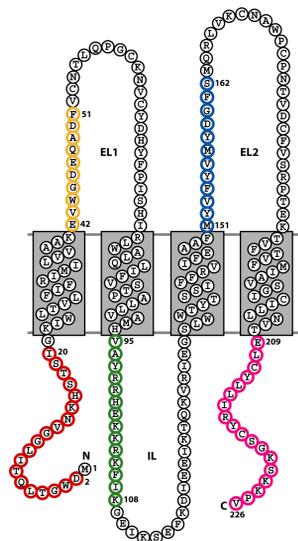
His-tagged, Cx26 was engineered to contain the single native cysteine in TM4. Protein was extracted from membranes and purified in each of the tested detergents. An aliquot (10 µg) of concentrated protein was diluted into 50 mM phosphate buffer pH7.5, 1.0 M NaCl, 2x CMC of detergent and the CPM fluorescent dye [7-deethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin] (Invitrogen). Samples were then heated at 2°C per minute in a Cary Eclipse spectrofluorimeter. Dye fluorescence was monitored using excitation and emission wavelengths of 387 and 463 nm, respectively. Four measurements were made for each detergent. A representative trace of the average raw fluorescence with standard error is shown (left graph).

Results:

- Cx26 was relatively stable in all detergents, with an average midpoint of the thermal transition of ~55°C.
- However, there were measurable differences, with ~5°C separating the extremes (inset at the right).
- The aliphatic chain of dodecylmaltoide (DDM) is one carbon longer than UDM, conferring ~2°C of additional stability.
- Among the additives combined with DDM, lipid reduced the transition temperature ~1°C, while both foscholine 14 (FC14) and cholesterol hemisuccinate (CHS) increased the stability by ~2°C.
- These measurements are being expanded to guide starting conditions for crystallization.

*Alexandrov, A.I., Mileni, M., Chien, E., Hanson, M., Stevens, R.C. Microscale fluorescent thermal denaturation assay to monitor stability of membrane proteins. In preparation.

Expression screening: Truncations in the M2-M3 cytoplasmic loop (IL) and EL2 are well tolerated



Membrane topology of connexins: Members of the connexin gene family contain four transmembrane domains (M1-M4), two extracellular loops (EL1 and EL2), a cytoplasmic loop (IL) between M2 and M3, and N and C-termini. A hexameric hemichannel, also called a connexon, docks end-to-end with a second hexamer to form the intercellular channel.

It is likely that modifications of wildtype, full length Cx26 will be required to achieve a high resolution crystal structure. The full length construct was used as a starting point for an extensive mutagenesis and expression screen.

Truncations of the extramembrane loops and tails:

Using the topology of Cx26 as a guide (left), we examined the expression of systematic truncations. Overall, 96 truncation mutants were attempted, consisting of single amino acid deletions of the N-terminus (red), C-terminus (purple), EL1 (yellow), EL2 (blue) and the M2-M3 IL (green). Small-scale baculovirus-mediated expression in SF9 cells was tested using a Vertiga-IM shaker/incubator (Thompson). Samples were solubilized with DDM, and the relative expression level was estimated by Western immunoblot analysis (above).

Results:

- There is robust expression of wildtype Cx26 in SF9 cells, and some truncation mutants displayed even higher levels of expression.
- Truncations at the N- or C-termini, and to a lesser extent the EL1 loop, resulted in no recombinant virus or a reduction of expression.
- Truncation of the cytoplasmic M2-M3 loop (IL) and EL2 were surprisingly well tolerated.
- Selected truncations involving both termini and IL have been expressed and purified at the 1 L scale, yielding milligram quantities of purified protein.
- The relative expression levels observed in the small-scale analysis have translated well to scale-up to one liter.

Hanson, M.A., Brooun, A., Baker, K.A., Jaakola, V.P., Roth, C., Chien, E.Y., Alexandrov, A., Velasquez, J., Davis, L., Griffith, M., Moy, K., Ganser-Pornillos, B.K., Hua, Y., Kuhn, P., Ellis, S., Yeager, M., Stevens, R.C. *Protein Expr. Purif.* (2007) 56(1):85-92.

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